ORIGINAL ARTICLE

Postantibiotic effects with *Bacteroides fragilis* determined by viable counts and CO_2 generation

Margrét Valdimarsdóttir[1,2], *Helga Erlendsdóttir*[1] *and Sigurdur Gudmundsson*[2,3]

[1]Departments of Clinical Microbiology, Borgarspitalinn (Reykjavik City Hospital), Reykjavik, Iceland; [2]University of Iceland Medical School, Reykjavik, Iceland; [3]Department of Medicine, Landspitalinn (University Hospital), Reykjavik, Iceland

Objective: To study the postantibiotic effect (PAE) for *Bacteroides fragilis* after exposure to common anaerobic antimicrobials with two different methods, by viable counting and by measuring CO_2 generation in a BACTEC® blood culture system.

Methods: Four strains of *B. fragilis* were exposed for 1, 2 and 4 h to cefoxitin, chloramphenicol, clindamycin, imipenem or metronidazole at concentrations from 1 to 16 × MIC. The drugs were removed by dilution into BACTEC 7A® vials and growth determined with viability counts and CO_2 production.

Results: The durations of the PAEs determined by the two methods correlated well (r=0.913, p<0.005). PAEs of up to 4–5 h were induced by imipenem and metronidazole with achievable concentrations and exposure durations. Chloramphenicol induced short or no PAEs, but cefoxitin and clindamycin induced PAEs up to 2 h with high AUC values. The imipenem PAEs and the short cefoxitin and clindamycin PAEs were dependent on AUC.

Conclusions: Significant PAEs against *B. fragilis* were induced by imipenem and metronidazole. Determining PAE by measuring CO_2 production is an accurate and less time-consuming alternative to the conventional method of viable counts.

Key words: Postantibiotic effect, CO_2 production, *B. fragilis*, cefoxitin, chloramphenicol, clindamycin, imipenem, metronidazole

INTRODUCTION

Anaerobic organisms comprise the dominant part of our normal flora, but are also recognized as common causes of serious infections at virtually all anatomic sites, particularly in the abdomen, lungs, pelvis, mouth and central nervous system [1]. Anaerobes are often found with aerobic or micro-aerophilic organisms in abscesses. Treatment of these infections with antimicrobials can be difficult, partly because of the special environment in the abscess.

While the postantibiotic effect (PAE) has been found to be a feature of all aerobic organisms tested so far [2], limited information is currently available on the PAEs of anaerobic organisms. In limited previous studies, β-lactam drugs were demonstrated not to induce a PAE against Gram-negative anaerobic bacilli, whereas clindamycin, chloramphenicol and, in particular, metronidazole induced PAEs [3].

We have previously demonstrated that PAEs of aerobic bacteria can be conveniently determined by measuring CO_2 generation in a BACTEC® blood culture system [4]. The BACTEC® method of following bacterial growth for PAE determination is simple and is less labor-intensive than the conventional method of viable counts.

We therefore determined the PAEs of several antimicrobial agents against four strains of *Bacteroides fragilis* with the BACTEC® NR-730 blood culture system, and compared them with PAEs determined in the standard fashion by viable counting in an anaerobic workstation.

Corresponding author and reprint requests:

Sigurdur Gudmundsson, Department of Medicine, Landspitalinn (University Hospital), 101 Reykjavik, Iceland

Tel: 354-560 1000 Fax: 354-560 1298

E-mail: siggudm@rsp.is

Accepted 30 August 1996

METHODS

Organisms

The study organisms were four strains of *Bacteroides fragilis*, three clinical strains, obtained from the Clinical Microbiology Laboratory at Borgarspitalinn, Reykjavik (B1, B2, B3), and a standard strain, ATCC 25285 (B4).

Antibiotics

The antibiotics used were: cefoxitin (Merck, Sharp & Dohme, Rahway, New Jersey, USA), chloramphenicol (Parke-Davis, Pontypool, UK), clindamycin (Upjohn, Kalamazoo, Michigan, USA), imipenem (Merck, Sharp & Dohme, Rahway, New Jersey, USA) and metronidazole (Icelandic Pharmaceuticals Ltd, Reykjavik, Iceland). The antibiotics were obtained as the standard powder, dissolved as recommended by the manufacturer and stored at −18 °C until diluting into the desired concentration before each experiment. MICs were determined by E-test® (AB Biodisk, Solna, Sweden).

Media

The organisms were grown overnight in BACTEC 7A® blood culture vials (Becton Dickinson & Co., Sparks, MD) containing 30 mL of prereduced soybean-casein digest broth to a slow logarithmic growth of ~0.2 $\log_{10}$ CFU/mL/h. The organisms in an inoculum of ~5×10^6 CFU/mL were exposed to the antibiotics in a prereduced general nutrient broth (heart infusion broth, Difco, Detroit, MI). After drug removal, BACTEC 7A® vials were again used for culturing. For viable counts the organisms were grown on prereduced 5% horse blood agar (Department of Microbiology, Landspitalinn (University Hospital), Reykjavik).

Exposure and drug removal

The organisms were exposed to the antibiotics under anaerobic conditions (Anaerobe systems, Shel-Lab, Cornelius, OR, USA) in prereduced heart infusion broth for 1, 2 or 4 h to antibiotic concentrations from 1 to 16 × MIC. However, the concentrations used never exceeded the serum concentrations reached after recommended dosages of each drug. A total of 148 combinations of particular exposure time versus particular concentration and strain were tested. Several experiments were repeated up to four times on different days.

Drug removal after exposure was accomplished by 10^{-2}–10^{-3} dilution of exposed cultures and unexposed control cultures into fresh prewarmed BACTEC 7A® vials, the dilution employed for the highest exposure concentrations of 16 × MIC. To account for killing of the exposed organisms, two additional dilutions were made of unexposed culture (final 10^{-3},10^{-4}). The calculations were based on extrapolations from the two dilutions closest to the culture. The potential effect of residual subinhibitory concentration was examined with the use of an additonal 'drug control'. In experiments with exposure concentrations of 8 and 16 × MIC, similarly diluted unexposed organisms were inoculated in a fresh vial in which a 10^{-2} or 10^{-3} dilution of the highest drug concentration used had been made. A 'drug control' growing at the same rate as unexposed control decreased the likelihood of significant amounts of residual subinhibitory levels of drug affecting the growth of the organisms in the PAE phase.

Quantitation of the PAE

The BACTEC 7A® vials were incubated at 35.5 °C. At 2.5–4-h intervals, CO_2 production in the vials was measured using the BACTEC NR-730® system. The system detects bacterial CO_2 by infrared spectroscopy of the gas aspirated from the headspace of the blood culture vial. Absorption of CO_2 by the spectrophotometer is expressed in terms of 'growth values' (GV), which is a unitless measure derived from comparison made by the system between the amount of CO_2 present in the vial headspace and the amount of CO_2 in aerobic (2.5% CO_2) or anaerobic (5.0% CO_2) reference 'culture gas'. The manufacturer recommends that 25–30 GVs be used as a 'cut-off' for subculturing a blood culture vial. The bacterial viable count corresponding to this number is variable, ranging from 10^6 to 10^8 CFU/mL, and depends on bacterial species, pH, oxygenation and temperature of medium, etc. (P. E. Goldenbaum, personal communication). In the PAE experiments, the bacterial growth in the vials was followed in this manner until a GV of 25 was reached.

In the first part of the study (for a total of 77 PAE determinations), 200–300-μL samples were obtained from the same vials just after the CO_2 measurement at 1.5–4 h intervals under anaerobic conditions. From these samples viable counts were determined by plating serial 10-fold dilutions of the samples on horse blood agar and incubating for at least 24 h under anaerobic conditions at 35.5 °C. In the remainder of the study all PAE determinations were done with the BACTEC® system.

The PAE was calculated according to the equation:

$$PAE = T - C \quad [1]$$

For the BACTEC® system, T is the time required for cumulative CO_2 production in exposed organisms to reach 25 GVs, and C is the time required for the untreated control culture to reach the same counts. For the viability method, T is the time required for the

count of CFUs in the test culture to increase 1 $\log_{10}$ above the count observed immediately after drug removal, and C is the time required for the count of CFUs in an untreated control culture to increase by 1 $\log_{10}$ above the count observed immediately after the identical removal procedure.

Analysis of data

The durations of the PAEs were expressed as means with 95% confidence intervals of the four strains tested, and replicate test results were averaged. Outlying results were handled by the criterion of Dixon [5]. Correlation of the PAEs by each method was determined by regression. The slope of the regression line was compared with a line with a slope of 1 by the t-test, and residuals from the regression line were evaluated by chi-squared test. The area under the concentration–time curve (AUC) was expressed as the product of the drug concentration in multiples of MIC and the time for which the test organisms were exposed to the drug ($\times$ MIC.h). The association between AUC and PAE was analyzed by univariate least-squares linear regression (SPSS version 6.0, SPSS Inc., Chicago, Ill.). An $\alpha=0.05$ was chosen as the level of significance.

RESULTS

Figure 1 represents examples of bactericidal activity after 1, 2 and 4 h of exposure to the antimicrobial agents studied at a concentration of 8 $\times$ MIC, and after exposure to the agents for 1 h at concentrations of 2, 4, 8, and 16 $\times$ MIC. Chloramphenicol and clindamycin exhibited static effects only.

As an example of typical regrowth curves after antimicrobial exposure, Figure 2 demonstrates regrowth (viability counting and BACTEC GVs) in an experiment involving *B. fragilis* ATCC 25285 after 1 h of exposure to metronidazole at a concentration of 8 $\times$ MIC. In this example, the duration of the PAE determined by the BACTEC method was 5.3 h and that by the viable count method was 4.2 h.

The correlation coefficient of the PAEs determined by the two different methods was $r=0.913$ ($n=77$, $p<0.005$, Figure 3). The regression line had an intercept of -0.11 ± 0.30 (95% confidence intervals) and a slope of 1.11 ± 0.12 (95% confidence intervals), which was not statistically different from a slope of 1.0. If the two methods were equivalent in determining the PAE, the slope would be 1.0 with an intercept of 0. The residuals about the regression line had a normal distribution without systemic deviation from the line, indicating that the two methods had equal variations. The mean difference in duration of the PAEs determined by the two methods was 0.9 ± 0.2 h (95% confidence intervals).

The MICs of the antimicrobial agents used and the duration of the PAEs induced against each of the *B. fragilis* strains after exposure to the agents at concentrations of 4 $\times$ MIC for 1 h are shown in Table 1. Considerable strain variation was demonstrated.

The relationship between the duration of the mean PAEs (with SE) and the corresponding AUCs of each

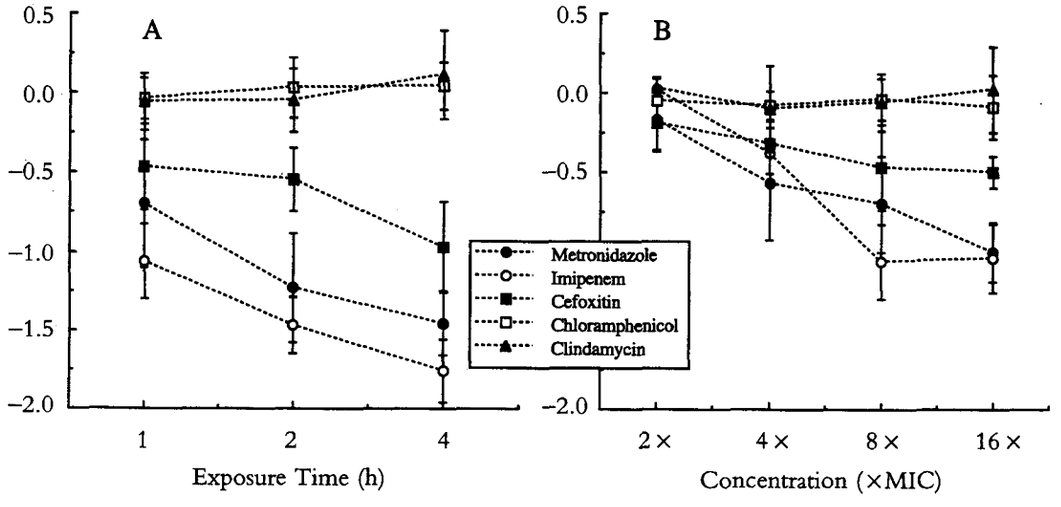

Figure 1 Examples of bactericidal activity against the four *B. fragilis* strains after 1, 2 and 4 h of exposure to metronidazole, imipenem, cefoxitin, chloramphenicol, and clindamycin at a concentration of 8 $\times$ MIC (A) and after 1 h of exposure to the agents at concentrations of 2, 4, 8, and 16$\times$ MIC (B) Vertical bars denote standard error of the mean.

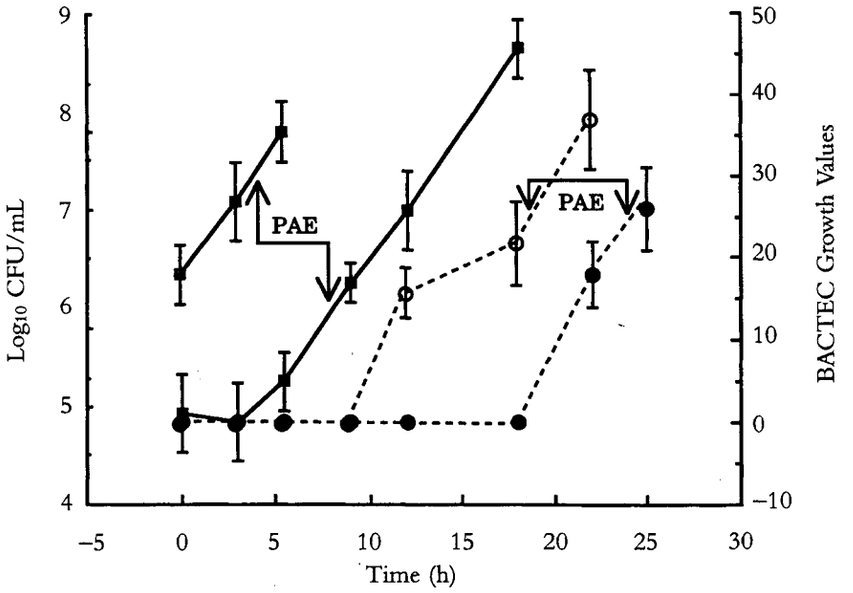

Figure 2 Regrowth curve of exposed and unexposed control organism (viability counting and BACTEC counts) from an experiment involving *B. fragilis* ATCC 25285 after 1 h of exposure to metronidazole at a concentration of 8 × MIC. Open symbols, unexposed controls; closed symbols, exposed organisms; square symbols, viability counts (log_{10} CFU/mL); round symbols, CO_2 generation (BACTEC growth values); arrows, times for calculating the PAE according to equation 1 (time *C* and *T*, see text).

drug is demonstrated in Figure 4. PAEs >1 h were induced by imipenem and metronidazole at AUCs easily attained in vivo. Cefoxitin and clindamycin induced PAEs > 1 h only at high AUCs, and chloramphenicol induced only very short or no PAEs at all

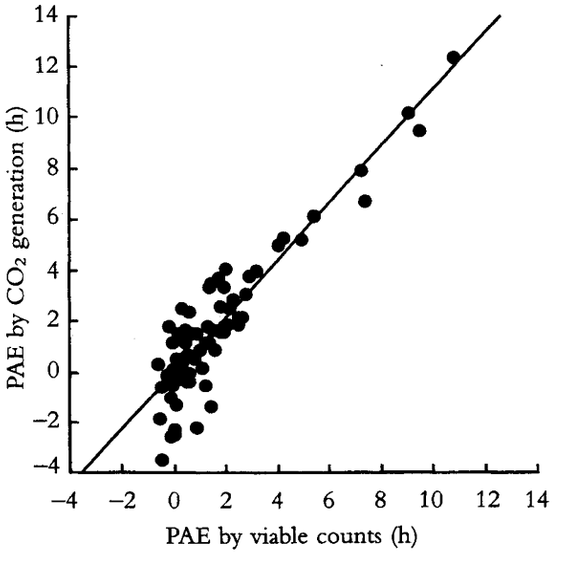

Figure 3 Correlation between the PAEs for all four strains of *B. fragilis* as determined by the viability count method and the BACTEC® method (n=77; r=0.913, p<0.005).

AUC values tested. The PAEs induced by the β-lactams imipenem and cefoxitin were consistently dependent on the AUC (slope (b)=0.05, p<0.0001, and b=0.07, p=0.001, respectively). Similarly, the short PAEs induced by clindamycin were dependent on AUC (b=0.03, p=0.02). In contrast, no significant correlation could be demonstrated for chloramphenicol in this regard (b=0.01, p=NS). A marginally significant linear trend of longer PAEs with increasing AUCs was observed for metronidazole with AUCs up to 8 × MIC.h (b=0.25, p=0.03), whereas a trend, albeit non-significant, of shorter PAEs with increasing AUCs of >8 × MIC.h was observed (b=−2.2, p=NS). For imipenem, cefoxitin and clindamycin, the duration of the PAE was dependent on both components of the AUC, drug concentration and duration of exposure (data not shown), whereas a different pattern was observed for metronidazole (Figure 5). Increased exposure concentrations of 4–8 × MIC were associated with prolongation of the PAEs, while increased exposure times even at identical drug concentrations were associated with reduced PAEs (p=0.02 for exposure concentration of 8 × MIC).

DISCUSSION

As demonstrated previously for aerobic organisms [4] these results demonstrate that the CO_2 generation

Table 1 MICs and PAEs of the four test strains after exposure to the antimicrobial agents at a concentration of 4 × MIC for 1 h. SE, standard error; ND, not done

Drug	Strains				Mean PAE (h±SE)
	B1	B2	B3	B4	
Cefoxitin					
PAE (h±SEM)	−0.1	0.1±0.1	−1.0	0.0	−0.2±0.2
MIC (mg/L)	1.5	4	2	1.5	
Chloramphenicol					
PAE (h±SEM)	0.7	0.4	−0.1	ND	0.3±0.2
MIC (mg/L)	2	1	1.5	1	
Clindamycin					
PAE (h±SEM)	ND	−0.1±1.2	−1.9±1.7	−0.2±1.0	−0.6±0.7
MIC (mg/L)	≤0.016	≤0.016	0.38	1.0	
Imipenem					
PAE (h±SEM)	0.4±0.1	1.2	0.0	1.1±0.1	0.7±0.2
MIC (mg/L)	0.19	0.25	0.125	0.125	
Metronidazol					
PAE (h±SEM)	1.2±0.3	7.4±1.8	3.8±0.5	1.4±0.2	3.9±0.8
MIC (mg/L)	0.125	0.5	0.25	0.25	

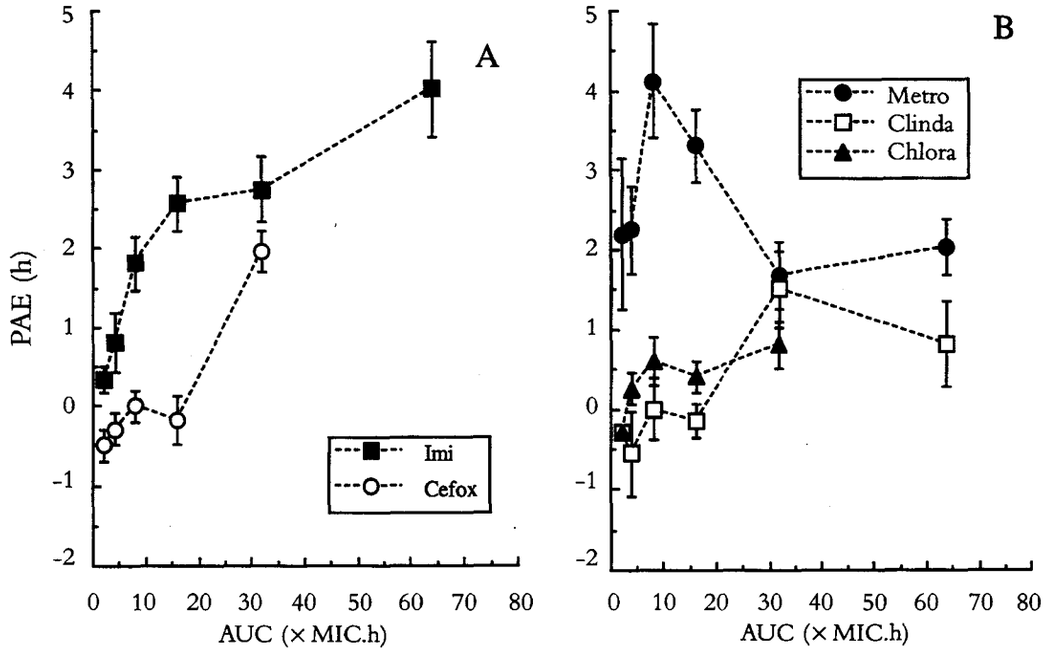

Figure 4 The relationship between the duration of the PAE and the AUC for all four strains. AUC is expressed as the product of exposure time in hours and drug concentration in multiples of MIC (× MIC.h). (A) β-lactam drugs, imipenem and cefoxitin; (B) non-β-lactam drugs, metronidazole, clindamycin and chloramphenicol. Vertical bars denote standard error of the mean.

method (BACTEC® method) can conveniently be employed for PAE determination of anaerobic organisms, the correlation with viable counting being satisfactory.

We exposed the organisms to the drugs under anaerobic conditions in the workstation where all samples were taken, while simultaneously determining the PAEs by viable counts and CO_2 generation. Viable counts of the exposed and unexposed control organisms need to be performed immediately after the drug removal procedure in order to adjust the number of organisms in the control growth to the number in

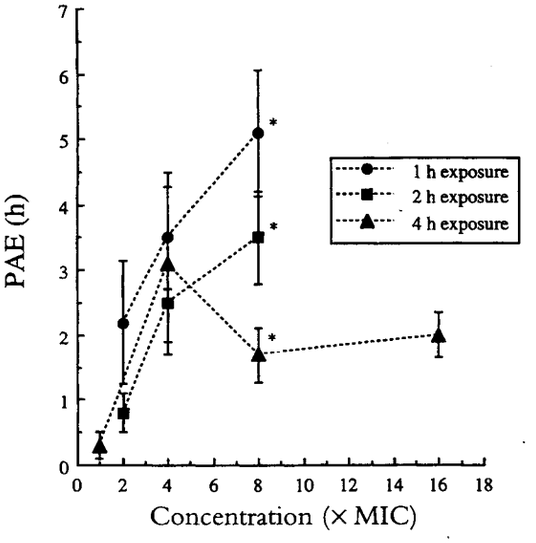

Figure 5 Relationship of mean PAEs for metronidazole to concentration in multiples of MIC and exposure time. Vertical bars denote standard error of the mean (*p=0.02).

the exposed growth and thus to account for the bactericidal activity.

Metronidazole, cefoxitin and imipenem exhibited concentration- and exposure-time-dependent killing, whereas only static activity was observed after chloramphenicol and clindamycin. In contrast, Klepser et al. recently demonstrated bactericidal activity of ~0.2–1.0 $\log_{10}$ CFU/mL over a 4-h period, dependent on exposure time but not concentration [6]. The discrepancy between these observations and ours of no bactericidal activity may be explained by strain difference and different exposure media.

PAEs of potential clinical significance of up to 4–5 h were induced by imipenem and metronidazole against the four strains of *B. fragilis* at achievable clinical concentrations and exposure durations. Similarly, Garcia-Rodriguez et al. have recently demonstrated a PAE for *B. fragilis* of 2–4 h after exposure to meropenem [7]. Carbapenems are unique among β-lactams in inducing PAEs against Gram-negative aerobic bacilli, particularly *Pseudomonas aeruginosa* [8–10]. Whether these properties are likewise unique with regard to *B. fragilis* needs further study. Long PAEs of 5–7 h have similarly been demonstrated for trospectomycin against *B. fragilis* [11].

In contrast, short (~1 h) or no PAEs could be demonstrated for chloramphenicol in this study, but PAEs for cefoxitin and clindamycin of up to 2 h were attained with high AUC values.

Similarly, Craig and Mattie [3] in their studies with strains of *B. fragilis* demonstrated no or short PAEs following exposures to cefoxitin, moxalactam and amoxicillin/clavulanic acid. Metronidazole produced long PAEs (6–7 h). However, in contrast to the results presented here, they demonstrated PAEs induced by clindamycin of 3–5 h and by chloramphenicol of 2–3 h. The reason for this discrepancy is not clear, but it could be due to strain differences encountered in this study (Table 1) as well as different growth media in these two studies.

The PAEs induced by imipenem and the short (probably clinically insignificant) PAEs induced by cefoxitin and clindamycin were dependent on AUC. A dependence of PAE on AUC has been described with other organisms and drugs, such as rifampin against *E. coli* and amphotericin C against *Candida albicans* [2,12]. For many drugs the PAE lengthens with higher values of AUC up to a point where increasing exposure time and drug concentration no longer affects the PAE, which stays at a plateau [12]. The PAEs induced by metronidazole were likewise dependent on AUC up to a maximal value, whereupon the PAEs got shorter with increasing AUC. This reduction was in particular related to increasing exposure times (Figure 5). This is difficult to explain but might be caused by down-regulation of receptors, an adaptive change in the metabolism of the bacteria, or some other mechanism at a cellular level [13].

In conclusion, determining PAEs of anaerobic organisms by measuring CO_2 production is an accurate, economic and less time-consuming alternative to the conventional method of viable counts. However, further studies need to be performed with other β-lactam agents and with other anaerobic organisms (particularly Gram-positive cocci).

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